Migration Assay for Improving Chronic Wounds

Elizabeth Borowiec
eborowiec9521@gmail.com

Taylor MacEwen
taytay1515@gmail.com

Lindsay Gardner
lindsay.gardner13@gmail.com

Kimberly Savitsky
ksav44@gmail.com

NJ Governor’s School of Engineering and Technology 2012

1. Abstract

An increasingly prominent medical condition that lacks sufficient treatment is chronic wounds. Chronic wounds are those which have failed to complete the normal wound healing cascade and therefore, are unable to close properly. These wounds frequently affect individuals with poor circulation and disease, such as diabetics, elderly, obese and bedridden patients, and cause them severe discomfort [1]. Mesenchymal stromal cells (MSCs), a type of multipotent stem cells, offer a potential solution. They secrete proteins, such as interleukin-8 (IL-8), which have the potential to facilitate accelerated wound healing. In a wound healing assay, an in-vitro system consisting of a monolayer of fibroblasts cultured on a surface was scratched to mimic a wound. The scratch was monitored over a 48 hour period to observe the migration of the fibroblasts in the monolayer. This wound healing assay allows for the observation of the migratory effects of culturing fibroblasts in mesenchymal stromal cell conditioned media (MSC-CM) in comparison to the control medium, Dulbecco’s modified eagle medium (DMEM). It was predicted that the fibroblasts cultured in 48-hour MSC-CM would migrate to close the simulated wound the fastest; in theory, the 48-hour MSC-CM would emit the most proteins, consequently nourishing the cells and inducing migration. Contrary to what was expected, the dermal fibroblasts that closed the simulated wound the fastest were those that were treated with the MSC-CM that MSCs were cultured in for 24 hours. However, the skin cells treated with the MSC-CM in which the stem cells had been cultured for 48 hours moved the fastest, although they could not close the wound before the fibroblasts cultured in the 24-hour MSC-CM. Essentially, the rate at which the fibroblasts migrated concurred with the expected hypothesis, though the results merely displayed which wound closed first.

2. Key Terms

**Dermal Fibroblasts**: the skin cells used to create the monolayer of cells used in the scratch assay; They migrate to close wounds and form granulation tissue in the proliferation stage of wound healing.

**Mesenchymal stromal cells (MSCs)**: multipotent adult stem cells found in bone marrow; these stem cells can secrete nutrients, such as proteins that have the potential to increase cell growth to heal chronic wounds.

**Mesenchymal stromal cell-conditioned media (MSC-CM)**: media that has been used to culture MSCs; the factors that MSCs
secrete are dissolved in the media; this media is considered conditioned by the MSCs and can now be used to culture other cells; newly cultured cells benefit from the factors in the secretions in the media.

**Leukocytes:** white blood cells

**Cytokines and chemokines:** proteins and chemicals secreted by a variety of cells that cause a response from other cells

**Neutrophil:** a type of white blood cell that defends against fungal and bacterial infections

**Macrophage:** a type of white blood cell; monocytes become macrophages when they enter tissue; the main purpose of a macrophage is to clean cell debris away.

3. **Introduction**

When there is an injury to the skin, a series of steps occur to heal the wound in a timely and efficient manner. The purpose of this tightly regulated process is to restore the former integrity of the skin. Although wounds heal differently depending on various circumstances, each progresses through four specific phases: the immediate response, inflammatory response, proliferation phase, and remodeling phase.

3.1 **The Immediate Response**

Immediately after the skin suffers a break in its integrity, physical and chemical signals change, thus altering the metabolism and gene expression to heal the wound. Vascular vessels constrict to prevent hemorrhaging while platelets aggregate and activate to form a fibrin clot. This clot blocks blood flow out of the wound while acting as an area where growth factors can bind and cells can migrate [3].

3.2 **The Inflammatory Response**

After the platelets form the fibrin clot, circulating leukocytes migrate from the bloodstream into the wound. These immune cells in the wound secrete different cytokines and chemokines that recruit neutrophils and macrophages. Neutrophils destroy invading microorganisms to prevent infection throughout the healing process. Macrophages clear away the cell matrix and cellular debris, including spent neutrophils. Angiogenesis, the second stage of the inflammatory response, is the process that reforms capillaries around the wound to provide necessary nutrients [3].

3.3 **The Proliferation Phase**

Wound closure occurs during the proliferation phase of wound healing. The wound attempts to restore as much of the integrity of the skin as possible with regards to its elasticity and strength. At this time, dermal fibroblasts migrate towards the center of the wound, forming the soft pink granulation tissue characteristic of newly healed wounds. Granulation tissue eventually replaces the fibrin clot formed in the immediate response. The keratinocytes, a specialized type of skin cells that act as a protective barrier, migrate towards the center of the wound and seal the edges [3].

3.4 **The Remodeling Phase**

However, the healing process is not over simply because the wound is closed. In order for the skin to return to full functionality, the fibroblasts synthesize collagen, a major component of scar tissue. The collagen fibers cross link to enhance the strength of the scar. This process continues well after the wound has re-epithelialized. Additionally, blood vessels in the dermis
mature to form a working network to the new scar tissue. Once the wound is sealed, and all integrity is restored to the skin, the wound is completely healed [3].

3.5 Chronic Wounds

If the wound does not follow the natural wound healing cascade described above, it fails to close and becomes a chronic wound. A wound is more likely to become chronic if the patient is bedridden, obese, diabetic, or elderly. Each of these conditions decreases blood flow to wound sites, depriving the wound of oxygen, growth factors, and other nutrients essential to wound healing. Most patients who receive treatment for chronic wounds are already hospitalized for other conditions; henceforth, chronic wounds have been considered a comorbidity and were never classified as their own ailment until recently. Because several factors contribute to the formation of chronic wounds, many considerations must be taken into account when treating the wound. Consequently, chronic wound care is costly in many aspects, namely financially, physically, and emotionally, as some chronic wounds result in amputation, especially for diabetics. Approximately 25% of all diabetics will develop a foot ulcer, a type of chronic wound, and about half of these will end in amputation. Chronic wounds have gained notice as a medical issue of great concern because 6.5 million Americans suffer from them [1]. Worldwide, the number of obese, diabetic and elderly people has dramatically increased. This spike has caused a wider pool of patients to be at risk for chronic wounds and more money to be spent on chronic wound treatments. Estimates show that more than $25 billion is spent annually on chronic wound treatments worldwide. As a result, studies to discover the causes of chronic wounds have been conducted to improve patient health [4].

3.6 Current Advanced Treatments and Healing Techniques

The typical wound healing treatment process consists of cleansing and regular monitoring of the wound. The initial step is to remove any wound healing deterrents like bacteria or necrotic material that could impede the normal healing cascade. The goal of wound care is to preserve the vital tissue while removing any impeding material. Presently, there is no distinct evidence that proves one form of wound healing to be more helpful or beneficial than another in terms of decreased healing time. Inflammatory mechanisms that become uncontrollable and self sustained commonly contribute to wound healing failure, a factor leading to chronic wounds [5]. In order to minimize the potential for additional trauma and cytotoxicity, wounds should be cleansed in biocompatible solutions. The wound bed should typically be moderately moist but not macerated. A wound that is too moist could instigate the potential for bacterial infection; on the other hand, if the wound is too dry, it takes longer to heal.

Chronic wound healing methods today have yet to be perfected. Unfortunately, the average education time spent on wound healing and physiology in American medical schools is 0.7 hours in 4 years [1]. Doctors should receive far more in depth chronic wound education, specifically in their treatment, thereby developing the skills to recognize, potentially prevent, and heal them. Until further research is performed in this field, doctors must optimize the current, yet lacking, wound healing techniques. A large array of therapeutics exists, and current treatments and procedures have progressed over the past decades. However, the demand for research is fortified as chronic wounds gain prominence. One such treatment that necessitates research is intermittent pneumatic compression (IPC) for chronic
venous ulcers. This technique uses a simple compression therapy that includes an application of 20-120 mmHg of pressure to the wound site in hopes of improving blood flow to increase skin cell migration. Even so, it requires that a patient be subjected to treatment for about two hours daily for two weeks. Patient compliance becomes an issue as it is uncomfortable to be attached to a bulky device for that time period. Another form of treatment lies in hyperbaric oxygen. Most chronic wounds involve a severe oxygen deficiency at the wound site, formally called hypoxia. Although applying 100% pressurized oxygen onto the wound could be effective for some, the treatment is only specialized for specific patients and requires high expertise and expensive equipment. Hence, the impact on patient care is fairly limited.

Other forms of treatment for chronic wounds include biosurgery, drugs, and tissue engineered skin substitutes. Biosurgery utilizes sterile maggots to remove slough and necrotic tissue. While it is cost-effective, the maggots required for the procedure have a short life span. In addition, a lack of aesthetic appeal and increased pain at the wound site increase uncertainty regarding this treatment choice. Drugs are praised for their ability to treat patients without invasive processes, though drugs currently on the market have only provided relief for a fraction of all patients suffering from chronic wounds. Moreover, proper medication to treat chronic wounds does not exist [6]. Another current chronic wound treatment option that has shown some beneficial effect is the use of tissue engineered skin substitutes. However, the use of skin substitutes is limited by their high costs, susceptibility to infection, and in some instances, variable take rates. An autologous skin graft may be used alone or in combination with tissue engineered skin. However, this option is not often used because to create a graft, skin must be taken from a different area of the patient’s body, therefore creating another wound. In high risk patients, this wound has a great potential to become chronic [7]. There is potential for skin grafts to be donated from other sources, but this method has an incompatibility risk as well. The high risk of complication involved with skin grafts makes them scarcely used. As a result, despite the ability to treat chronic wound patients in the modern world, finances, efficacy, and availability continue to be an issue. For patients living with chronic wounds, a life of limited mobility is at hand, which highlights the necessity and significance of the work to improve treatments.

3.7 Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) are adult tissue stem cells located in bone marrow. These multipotent cells can differentiate into a variety of cell types, including bone, cartilage, and fat cells. However, in this experiment, the MSCs are used because of the soluble factors they secrete rather than for their differentiation capabilities. Prior research has shown that the factors MSCs secrete are therapeutic in the tissue repair process and enhance fibroblast growth and migration during wound healing.

In this study, the potential benefit of MSCs on wound healing was studied by investigating their effect on fibroblast migration in a wound healing assay in vitro. It was predicted that in this assay, fibroblasts cultured in MSC-CM would migrate to close a simulated wound in a monolayer of cells faster than fibroblasts in the control medium, 0.1% FBS DMEM. Factors secreted by the MSCs may be responsible for their effects on fibroblasts because the secreted proteins can provide a surplus of nutrients and
stimulants for the cells, thereby expediting fibroblast migration [2]. It was expected that the fibroblasts in the MSC-CM that was conditioned for 48 hours would migrate more rapidly than those in the MSC-CM that was cultured for only 24 hours. This hypothesis stemmed from the notion that the longer culture time frame would correspond to a more substantial quantity of proteins emitted from the MSCs.

4. Experiment

4.1 Culturing Dermal Fibroblast Monolayer

Human dermal fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) until the cells reached 90-100% confluency [8]. Once the fibroblasts reached confluency, the DMEM was aspirated from the cells, and 8 mL of trypsin was added to enzymatically detach the cells from the flask. Once detachment occurred, the trypsin was deactivated using 8 mL of DMEM. The fibroblast solution was then transferred to a test tube and placed in the Allegra X-22R centrifuge to separate the media and the cells. The DMEM and trypsin solution was aspirated from the fibroblast pellet, and the cells were re-suspended in DMEM. The cells were then counted using a hemacytometer and a CKX41 inverted light microscope. The following equation was utilized to calculate the fibroblast concentration: (# of cells counted in sample/4)*dilution factor*10,000 cells/mL = \( C_1 \). Once \( C_1 \) was calculated, the dilution equation for solutions \( C_1V_1 = C_2V_2 \) was used to find the volume of fibroblast solution necessary to ensure a final cell concentration of 100,000 cells/mL. This concentration of fibroblasts was added to 6 wells in a 24-well plate in 1 mL of media. The cells were then incubated overnight to allow cell attachment to the well plate.

On the underside of the well plate, black boxes were drawn with a thin marker in eight wells. This strategy allowed for the observation of the same section of the well each time. Once the region was constricted, a scratch was made using a 1.5mm pipette tip. The scratch represents the proliferation phase of the wound healing cascade; as the fibroblasts begin the migration and actually close the wound. The DMEM was aspirated from each well to remove any dislodged cells, and 1 mL of media containing treatment was added to each well. The treatments included DMEM containing 0.1% FBS, DMEM containing 10% FBS, or mesenchymal stromal cells conditioned media (MSC-CM) that was conditioned with MSCs for 24 hours or 48 hours in DMEM containing 0.1% FBS.

The cells were photographed at 0, 6, 18, 24, 42, and 48 hours using an inverted light microscope attached to a Nikon camera. The images were analyzed using an NIH imaging software, ImageJ. The images were uploaded onto the program so that measurements of the width of the wound could be analyzed. Three points on the incision (top, middle, and bottom) were used to gain numerical widths, which were averaged and later converted into micrometers. The wound widths were normalized to the original wound size at time 0 and graphed [2].
4.2 ELISA: Determination of IL-8 Concentration in MSC DMEM

In order to analyze the MSCs effect on dermal fibroblast migration, an assay was performed to measure protein concentration in the MSC-CM. This enzyme-linked immunosorbent assay (ELISA) is a standard procedure conducted to determine the exact concentration of a protein present in a
solution. In this experimentation, the ELISA was specifically conducted to record the concentration of interleukin-8 (IL-8), a chemokine secreted by MSCs. IL-8 is a protein of interest due to its ability to maintain homeostasis in cell migration during healthy tissue development. Previous research showed that IL-8 from human multipotent stromal cells increased fibroblast migration \[9\]. In fact, MSC-conditioned media was found to accelerate dermal fibroblast migration \textit{in vitro} \[10\].

IL-8 capture antibody (0.1 mL) was added to a 96-well plate and incubated overnight at 4 degrees Celsius. The following day, the wells were washed four times with a wash buffer, and 0.2 mL of a blocking solution was added to the wells for 1 hour. Then, the wells were washed, and 0.1 mL of diluted standards or samples was added to each well and incubated for two hours on the plate shaker. Washing was repeated four more times, 0.1 mL of antibody detection was added to each well, and the plate was incubated on a plate shaker for one hour. The wells were washed four times, 0.1 mL of Avidin-HRP was added to the wells, and the plate was incubated on the plate shaker for 30 minutes. Finally, the plate was washed five times, 0.1 mL of substrate solution C was added to each well, and the plate was incubated in the dark for 15 minutes in order to minimize the reaction of light with the solution, which would bleach the color and produce inaccurate results. Finally, 0.1 mL of the 2N sulfuric acid stop solution was added to each well to terminate the reaction. The solution was placed in a spectrometer and read at 450 nm absorbance to analyze light absorbed by the sample to determine protein concentration \[11\].

5. Results

\textit{Graph 5.1: Trial I, Wound Closure in Various Treatments over 48 Hours}
5.1 Results

In accordance with the hypothesis, the fibroblasts cultured in MSC-CM migrated faster to close the simulated wound than did the samples that solely contained 0.1% FBS DMEM. Figure 5.2 shows the normalized wound width over a period of 48 hours. The 10% FBS DMEM in the blue and the 0.1% FBS DMEM in the green healed at about the same rate to about the same point. Neither wound with this condition closed. The 24-hour MSC-CM in the yellow had the second fastest rate of migration, although it was the first wound to close. The red points represent the 48-hour MSC-CM which maintained the fastest rate of migration. At the end of the 48 hours, the 24-hour MSC-CM cells had completely migrated to close the wound, resulting in a wound width of zero, while the other wounds still had yet to close. The rates at which the wounds healed in both DMEM samples were very similar as were the rates of both the MSC-CM samples. The wound healing in the media from the stem cells cultured for forty-eight hours showed slight improvements in comparison to the media without MSC-CM or in the MSC-CM cultured for 24 hours, presumably as a result of the proteins; however, that scratch was the last to close. The scratches were inconsistent because they were drawn manually, individually, and repeatedly to ensure a wide enough wound to monitor cell migration. The lines outside each scratch occasionally obscured the view of the wound, although they were intended to assure that the same part of the wound was photographed and measured in each trial. However, the results clearly demonstrated that although the cells in the 24-hour MSC-CM migrated to close the wound first, the most effective media for fibroblast stimulation and migration was the 48-hour MSC-CM because it induced the fastest rate of migration.

5.2: Comparison of IL-8 Secretion in Control Media and MSC-CM

We hypothesize that IL-8 is the compound derived from MSCs that stimulates dermal fibroblast migration. Therefore, the concentration of protein interleukin-8 (IL-8) was also analyzed. In comparison to the control medium without the stem cells’ secreted products, the media that did contain proteins secreted by the MSCs contained a significant amount of IL-8, as shown in Graph 4.2 below. This secretion of IL-8 is consistent with the hypothesis that mesenchymal stromal cells emit specific proteins that improved cell migration.

Graph 5.2: IL-8 in DMEM and MSC-CM

6. Discussion and Analysis

The results of this migration assay supported the hypothesis that the mesenchymal stromal cell conditioned media (MSC-CM) would expedite cell migration as compared to Dulbecco’s
Modified Eagle Medium (DMEM) with 0.1% Fetal Bovine Serum (FBS). It was expected that the media conditioned with MSCs the longest would promote cell migration the most because the MSCs would secrete more growth factors over a longer time period. However, the wound that closed the most rapidly was that which was cultured in the MSC-CM conditioned for 24 hours. This observation most likely occurred because the wound from the 48-hour-conditioned media was substantially larger than the simulated wound in the 24-hour-conditioned media (118 micrometers wider). Moreover, the wound in the 48-hour MSC-CM was only 81 micrometers larger than the wound in the 24-hour MSC-CM at the termination of the experiment. Another possibility is that the MSCs themselves began to use the proteins and nutrients after a 24 hour secretion time, which could have resulted in the discrepancy that the 24 hour closed first. The fibroblasts cultured in the 48-hour MSC-CM migrated at a faster rate than did those in the 24-hour MSC-CM. Theoretically, if the simulated wounds were identical at the start of the procedure, we expect that the wound in the 48-hour MSC-CM would have healed first.

These results only provide some insight into the properties of mesenchymal stromal cells. Further research can provide more information regarding their capability. For instance, this assay did not test the effects of a combination of MSC-CM and DMEM with a 10% FBS concentration on fibroblast migration. Although 10% FBS DMEM is traditionally used to culture dermal fibroblasts, this solution was avoided in this experiment because of its potential interference with other components in the MSC-CM. However, now that it has been established that the mesenchymal-conditioned media promotes migration, the combined effects of these two factors (10% FBS DMEM and MSC-CM) should be investigated. Additionally, the concentration of MSC-CM in the MSC-CM and DMEM solution can be varied to test how more or less of the MSC-CM alters the wound healing process.

Furthermore, it is not yet conclusively known what components of the MSC-CM contribute to cell migration. Therefore, additional testing is required to examine which proteins or other factors secreted by mesenchymal stromal cells cause rapid fibroblast migration. The ELISA detected the presence of the IL-8 in MSC-CM; however, it was not determined whether or not IL-8 was responsible for the induced cell migration in our system. Subsequent testing may be used to confirm or contradict this possibility. Adding purified IL-8 to 0.1% DMEM, as opposed to using a MSC-CM and DMEM solution, may determine whether or not IL-8 is a contributing factor or inhibitor of migration. If the results of the future IL-8 experiment demonstrate that IL-8 has an identical or faster effect on wound closure than that of MSC-CM, it may be concluded that IL-8 is the primary reason the cells move together faster than they do without MSC-CM. On the other hand, if the IL-8 conditioned cells move slower than the fibroblasts cultured in MSC-CM, then the IL-8 may not be the only contributing factor, may not contribute at all, or may even delay the process. However, it does not yet prove that IL-8 is the protein in question. To address this, it would be necessary to specifically block or remove the IL-8 secreted by the MSCs and verify that migration is no longer stimulated. Moreover, the concentration of IL-8 can be compared from the 24-hour MSC-CM to the 48-hour MSC-CM using the ELISA. This experiment could determine if the concentration of MSC-CM and other nutrients is different from depending on the time the media was conditioned for.
Culturing dermal fibroblasts and mesenchymal stromal cells requires a precise technique to allow for accurate incisions and therefore, reliable cell migration data. Two flawed techniques include the drawing of black boxes on the well plate to create an incision target and the scratch assay itself. After analyzing preliminary samples, it was evident that the boxes drawn were too thick and the incisions too small. As a result, for a scratch assay, a larger pipette of about 1.5 mm was preferred. As a lack of uniformity became an issue when analyzing the migration rates of the fibroblasts, larger and more prominent incisions had to be made repeatedly, thereby increasing human error in the results.

The results of this experiment support the hypothesis that a scratch representing a chronic wound can be healed more rapidly with the application of mesenchymal stromal cells. Secretions made by the MSCs will possibly be useful in synthesizing new treatments for the exponentially increasing population of chronic wound patients.

As this population increases, the necessity for improved chronic wound treatments gains urgency. Though stem cells are currently not FDA approved for regular treatment on humans outside of clinical trials, this experiment demonstrates there is a wealth of potential for stem cells to dramatically alter the wound healing process. After more research and development to improve wound healing and chronic wound care is conducted, such potentials will be revealed. While fetal bovine serum has been proven to ameliorate fibroblast migration for quicker wound healing, the amount present in the samples with MSC-CM was merely enough to keep the cells alive without producing a significant effect on the cells’ migration. From this, it can be concluded that the media from the mesenchymal stromal cells yielded faster cell migration.

7. Conclusion

After completing migration assays on dermal fibroblast cultures in the control 0.1% FBS DMEM and MSC-CM, the fibroblasts cultured in media conditioned with mesenchymal stromal cells closed the wound more efficiently. The simulated wounds conditioned in MSC-CM decreased in width at a greater rate than those in 0.1% FBS DMEM. Moreover, the wound in the 48-hour MSC-CM decreased in width more sharply compared to the scratch in 24-hour MSC-CM, even though the latter closed first. The rate at which the wound in 48-hour MSC-CM healed was much greater than the 24-hour MSC-CM culture. Thus, additional studies with regulated scratch widths may show the 48-hour MSC-CM to be the most effective in increasing fibroblast migration to close chronic wounds.

Stem cell treatments have the potential to drastically alter medical procedures regarding chronic wound care. Understanding the importance of MSCs and their effect on cell migration has demonstrated the possibility of significantly improved chronic wound treatments. Investing time in these new methods has proven to be worthwhile thus far. The increased cell migration in the presence of proteins emitted by mesenchymal stromal cells suggests that these cells can be applied to heal multilayered chronic wounds. Further research to determine specific proteins secreted by MSCs that expedite fibroblast migration (such as IL-8) should be conducted and its findings implemented into current chronic wound healing treatments. This information would be useful to better understand how MSCs promote their beneficial effects. In addition, it would enable implementation of quality control criteria to be applied on individual batches of MSCs, which is important to ensure reliability of the product.
Acknowledgements

We would like to thank Renea Faulknor, our research mentor, for being our guide throughout this project, as well as Melissa Przyborowski, who assisted us in the lab and with our paper. We would also like to thank Dr. Francois Berthiaume, PhD whose lab we were so fortunate to be able to work in. Much appreciation goes to Ashley So, our RTA and project mentor. Additionally, we are grateful to all the other RTAs, especially Stoyan Lazarov and the head counselor, Adrien Perkins, for editing this research paper. We would like to thank Jean Patrick Antoine, program director of the New Jersey Governor’s School of Engineering and Technology (GSET). Finally, we would like to acknowledge GSET, its program sponsors, and Rutgers University and the Department of Biomedical Engineering, without whom none of this would have been possible.

References


